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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup>:</b> <b>C12Q 1/68, C07K 13/00, 15/00, C07H 19/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 94/23066</b> <b>(43) International Publication Date:</b> 13 October 1994 (13.10.94)
<b>(21) International Application Number:</b> PCT/US94/03416 <b>(22) International Filing Date:</b> 29 March 1994 (29.03.94) <b>(30) Priority Data:</b> 08/039,982 30 March 1993 (30.03.93) US <b>(71) Applicant:</b> UNITED STATES BIOCHEMICAL CORPORATION [US/US]; 26111 Miles Road, Cleveland, OH 44128 (US). <b>(72) Inventor:</b> FULLER, Carl, W.; 3397 East Monmouth, Cleveland Heights, OH 44118 (US). <b>(74) Agents:</b> WARBURG, Richard, J. et al.; Lyon & Lyon, 34th floor, 611 West Sixth Street, Los Angeles, CA 90017 (US).		<b>(81) Designated States:</b> AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> USE OF EXONUCLEASE IN DNA SEQUENCING  <b>(57) Abstract</b>  Method for performing a DNA sequencing reaction in the presence of exonuclease I or exonuclease VII.		

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GA	Gabon				

DESCRIPTIONUse Of Exonuclease In DNA SequencingBackground of the Invention

This invention relates to improved methods for DNA sequencing.

Tabor and Richardson, U.S. Patent No. 4,795,699, and  
5 U.S. Patent 4,962,020, describe a method for DNA sequencing using T7 DNA polymerase. Such sequencing may be performed in the presence of magnesium or manganese ions. Both patents are hereby incorporated by reference herein.

10 Summary of the Invention

This invention relates to an improved method for sequencing DNA in which an exogenous single-strand specific exonuclease (i.e., one with no DNA polymerase activity, and which does not degrade double-stranded DNA)  
15 is provided in the sequencing reaction. Applicant has determined that provision of a small amount (about 1 - 50 units, where one unit is the amount of enzyme which catalyzes the release of 10 nmole of acid - soluble nucleotide from denatured DNA in 30 minutes at 37 degrees  
20 Celcius under standard conditions, see U.S. Biochemicals 1990 catalog, Cleveland, OH, hereby incorporated by reference herein) of such exonuclease activity increases the sensitivity of detection of particular DNA bands within a sequencing gel.

25 Without being bound to any particular theory, Applicant believes that the exonuclease may improve sequencing results by degrading excess primer and/or primer molecules weakly bound to the DNA template at sites other than the primary binding site. Such weakly bound  
30 primer can also prime extensions, resulting in the production of secondary or background sequences which interfere with the interpretation of the primary sequence.

Absence of background sequence is particularly important for situations where sequence bands must be interpreted by machine or interpreted-quantitatively.

Thus, in a first aspect the invention features  
5 performing a DNA sequencing reaction in the presence of a single-strand specific exonuclease, e.g., exonuclease I, or exonuclease VII of E. coli. Such DNA sequencing reactions generally involve those reactions described by Tabor and Richardson, supra, that is, the reaction  
10 includes one or more deoxyribonucleotides and one or more dideoxyribonucleotides, a DNA polymerase, and appropriate buffer conditions. Also included in this reaction are the DNA template to be sequenced and a primer. The conditions are suitable for primer extension along the DNA template  
15 until incorporation of a chain terminating agent, such as the dideoxyribonucleotides.

In a related aspect, the invention features a kit for use in DNA sequencing which includes reagents necessary for DNA sequencing such as dideoxyribonucleotides,  
20 deoxyribonucleotides, DNA primers and the necessary buffers, and an exonuclease lacking DNA polymerase activity, as described above.

This invention not only provides the advantage of preventing primers from giving ambiguous sequences, but  
25 also causes removal of excess primer after the initiation of a sequencing reaction. This removes the primer peak in a gel and allows sequence information to be obtained from the first few bases on the template immediately adjacent the primer binding site. Thus, an extra 6 - 10 bases can  
30 be read in such an experiment. This is particularly useful in automated sequencing reactions.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawing will first briefly be described.

Drawing

The figure is a graphical representation of DNA  
5 sequencing data obtained in the presence or absence of  
exonuclease I.

Referring to the figure, sequencing reactions were  
run generally as described in the SEQUENASE® Dye Primer  
sequencing kit protocol of A.B.I and U. S. Biochemical  
10 1992. Specifically, four annealing reactions were  
performed in reaction buffer (40mM MOPS pH 7.5, 50mM NaCl,  
10mM MgCl<sub>2</sub>, 15mM sodium isocitrate, and 5mM MnCl<sub>2</sub>) and  
dye-labeled universal (-21) M13 primers. The A and C  
annealing reactions contained 0.1 pmol of M13mp18 template  
15 DNA and 1.0 pmol of primer (FAM-labeled primer for C and  
JOE-labeled primer for A) in a volume of 4  $\mu$ l. The G and  
T annealing reactions contained 0.3 pmol of M13mp18  
template DNA and 3.0 pmol of primer (TAMRA-labeled primer  
for G and ROX-labeled primer for T) in a volume of 11  $\mu$ l.  
20 Thus a total of 0.8 pmole (2 $\mu$ g) of M13mp18 DNA was  
combined in four annealing reactions. Annealing was  
carried out by heating at 65°C for 2 min and slow cooling  
to <30°C ( about 35 min). Then termination mix (1000M  
dATP, dCTP, dTTP, 7-deaza-dGTP and 3.3M of respective  
25 ddNTP) was added to each reaction vial (1 $\mu$ l to the A and  
C reactions; 2 $\mu$ l to the G and T reactions, respectively)  
making the final concentration of nucleotide in the A and  
C reactions 167 $\mu$ M dNTP and 0.55 $\mu$ M ddNTP. Similarly, 133  
 $\mu$ M dNTP and 0.44  $\mu$ M ddNTP was used in the G and T  
30 reactions.

Stock enzyme contained SEQUENASE® Version 2.0 DNA  
Polymerase (13 Units/ $\mu$ l) and Inorganic Pyrophosphatase (12  
Units/ $\mu$ l). This stock was diluted 6-fold in Enzyme  
Dilution Buffer (10mM Tris pH 7.5, 0.1mM EDTA). Reaction  
35 vials were pre-warmed at 37°C. Then 1 $\mu$ l of diluted stock  
enzyme (2.2 units DNA polymerase) was added to each of the  
A and C reactions. Similarly, 2 $\mu$ l (4.4 units DNA

polymerase) was added to each of the G and T reactions. The mixtures were incubated for 20 min at 37°C. The four reaction mixtures were combined into one vial and 15 $\mu$ l of Stop/Salt solution (1M NaOAc, 20mM EDTA), and 180 $\mu$ l 95% ethanol added. After 15 min at -20°C, the precipitated DNA was collected by centrifugation (12,000xg 15 min), and washed twice with 70% ethanol (repeating centrifugation).

The precipitated DNA was resuspended in 5  $\mu$ l 83% deionized formamide containing 8.3 mM EDTA, heated at 95°C for 2 min and applied to an 8.3 M urea, 6% polyacrylamide gel mounted in the ABI 373A sequencing instrument. Electrophoresis was conducted at a constant 35W and 42°C for 14 hours. The data were collected and analyzed using the ABI software.

Referring to section (A) of the figure, the sequence of M13mp18 DNA with no Exo I used in the reaction mix is shown. The sequence shown is approximately 200 bases from the priming site. The upper (major) sequence is the one interpreted by the instrument software. Errors in this sequence are underlined. The lower (shaded) sequence is the complement of the sequence of M13mp18 from bases 4936-4903. This sequence aligns well with the apparent sequence represented by the background signals (smaller peaks) in this experimental run. It is the result of priming by the -21 primer at a weak site of homology at base 5077. The primary sequence is the result of priming at a 100% matched site at base 6291.

Referring to section (B) of the figure, the same DNA is shown sequenced as described above, but with a total of 3 units of Exo I added at the same time as the polymerase. The A and C reactions received 0.5 units Exo I while the G and T reactions each received 1 unit. Note that there are no errors in this sequence and that background peaks are eliminated or greatly reduced in intensity. There is no identifiable secondary sequence and the accuracy of the sequence obtained is much higher.

Other embodiments are within the following claims.

Sequence Listing

## (1) GENERAL INFORMATION:

5 (i) APPLICANT: Carl W. Fuller

(ii) TITLE OF INVENTION: USE OF EXONUCLEASE  
IN DNA SEQUENCING

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

10 (A) ADDRESSEE: Lyon & Lyon  
(B) STREET: 611 West Sixth Street  
(C) CITY: Los Angeles  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 90017

(v) COMPUTER READABLE FORM:

15 (A) MEDIUM TYPE: 3.5" Diskette,  
1.44Mb storage  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: IBM MS-DOS (Version  
5.0)

20 (D) SOFTWARE: WordPerfect  
(Version 5.1)

(vi) CURRENT APPLICATION DATA:

25 (A) APPLICATION NUMBER: 08/039,982  
(B) FILING DATE: March 30, 1993  
(C) CLASSIFICATION: 435

(vii) PRIOR APPLICATION DATA:

Prior applications total,  
including application  
described below: None

30 (A) APPLICATION NUMBER:  
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

35 (A) NAME: Warburg, Richard J.  
(B) REGISTRATION NUMBER: 32,327  
(C) REFERENCE/DOCKET NUMBER: 200/013

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(B) TELEFAX: (213) 955-0440  
(C) TELEX: 67-3510

## 5 (2) INFORMATION FOR SEQ ID NO.: 1:

## (i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 35  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

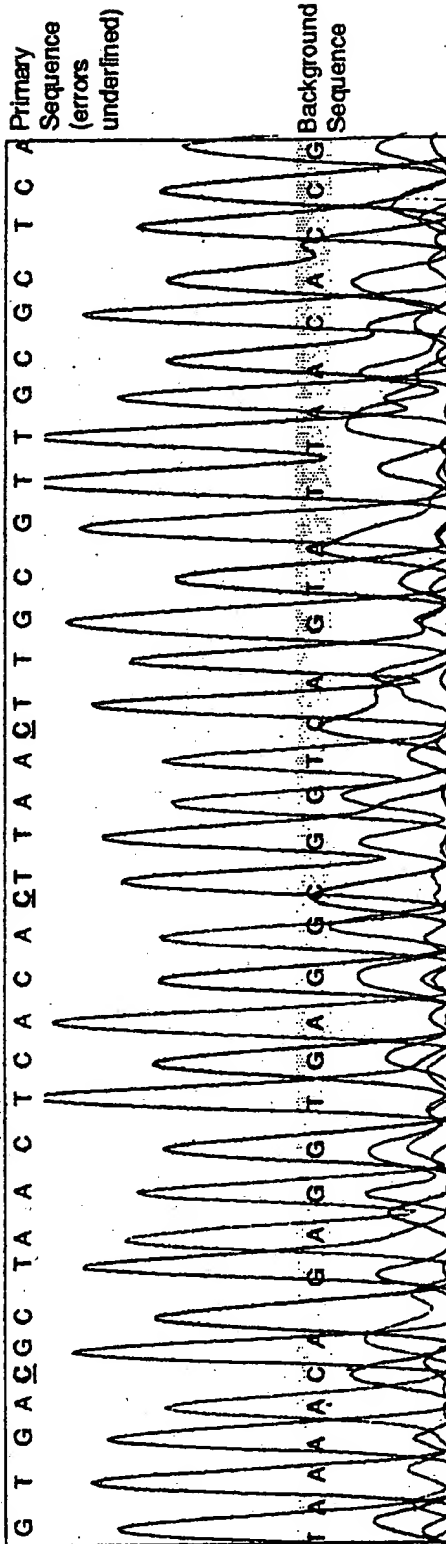
GTGAGCTAAC TCACATTAAT TCGTTGCGC TCACT 35



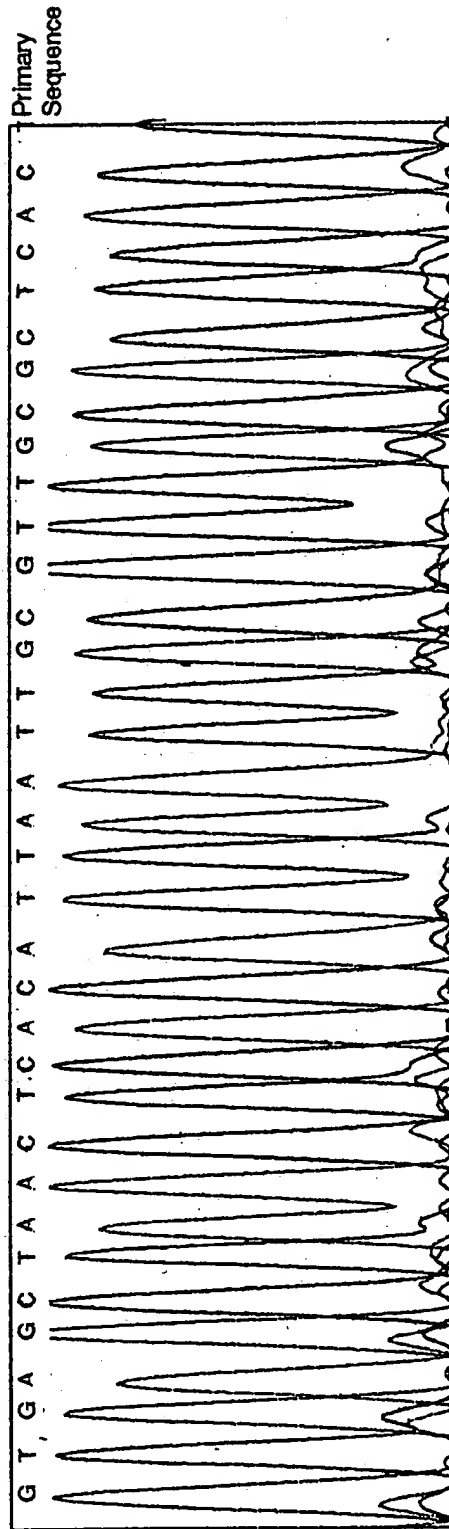
Claims

1. Method for performing a DNA sequencing reaction in the presence of a single-strand specific exonuclease lacking DNA polymerase activity, comprising the steps of:  
5           contacting a DNA template and primer with a DNA polymerase, at least one deoxyribonucleotide, a chain terminating agent, and said exonuclease under primer extension conditions.
2. Kit for use in DNA sequencing comprising in  
10 separate containers one or more reagents necessary for DNA sequencing including a dideoxyribonucleotide, and a single-strand specific exonuclease lacking DNA polymerase activity.
3. The kit of claim 2, wherein the exonuclease is  
15 exonuclease I.
4. The method of claim 1 wherein said exonuclease is exonuclease I.
5. The method of claim 1 wherein said exonuclease is exonuclease VII.
- 20 6. The kit of claim 2 wherein said exonuclease is exonuclease VII.

## A. Sequence without Exonuclease I



## B. Sequence with 3 Units Exonuclease I



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/03416

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C07K 13/00, 15/00; C07H 19/00

US CL :435/6; 530/350; 536/22.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 530/350; 536/22.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, MEDLINE, BIOSIS, WPI

search terms: sequencing, single-strand exonuclease, polymerase, exonuclease I, exonuclease VII

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Stratagene Catalog, issued 1988, "Gene Characterization Kits", page 39.	2, 3
Y	US, A, 4,795,699 (TABOR ET AL.) 03 January 1989, column 5, lines 17-60.	1-6
Y	Journal of Biological Chemistry, Volume 253, No. 2, issued 25 January 1978, K. R. Thomas et al, "Processivity of DNA Exonucleases", pages 424-429, especially page 428, third full paragraph.	1-4
Y	Journal of Biological Chemistry, Volume 249, No. 14, issued 25 July 1974, J. W. Chase et al, "Exonuclease VII of Escherichia coli. Purification and Properties", pages 4545-4552, especially page 4549, paragraph bridging columns 1 and 2.	1, 2, 5, 6

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

13 JUNE 1994

Date of mailing of the international search report

JUN 27 1994

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